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APPLICATION DATA SHEET

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Title of Invention	Prophylaxis of Respiratory Syncytial Virus Infection with Intranasal siRNA-Nanoparticles of the NS1 Gene

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Title of Invention	Prophylaxis of Respiratory Syncytial Virus Infection with Intranasal siRNA-Nanoparticles of the NS1 Gene

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Date :

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Comments

Prophylaxis of Respiratory Syncytial Virus Infection with Intranasal siRNA-Nanoparticles of the NS1 Gene

DESCRIPTION

[Para 1] BACKGROUND OF INVENTION

[Para 2] RSV is a major viral respiratory pathogen and produces an annual epidemic of respiratory illness causing bronchiolitis and otitis media in infants and young children1, 2 and pneumonia in adults and elderly 3, 4. The number of deaths attributable to RSV infection in the United States has been recently estimated to be over 17,000 per year5, RSV is also a major risk factor for a number of other health conditions such as immunodeficiency, cardiac arrhythmia, congenital heart disease6-8. An effective vaccine is not available for RSV and may not even be practical given the immunocompromised state of the target high-risk population, the incomplete immunity developed even by natural RSV infection, and its short incubation period9, 10. Consequently, DNA-based prophylactics are under investigation.

[Para 3] RSV is the prototypic member of the genus Pneumovirus and is an enveloped, nonsegmented, negative-stranded RNA virus. The RSV genome of approximately 15,200 nucleotides is transcribed into 10 transcripts encoding 11 distinct proteins, including two nonstructural proteins, NS1 and NS2, which are expressed from separate mRNAs encoded by the first and second genes, respectively11. Deletion of either NS1 or NS2 severely attenuates RSV infection in vivo and in vitro, indicating that NS proteins play an important role in viral replication12-15. Furthermore, repeated RSV infections are common due to the incomplete immunity caused by natural infection, the basis for which is poorly understood16. Clinical studies demonstrate that RSV infection in infants is associated with a predominantly Th2-like response17. Hence, RSV is considered a predisposing factor for the development of allergic diseases and asthma18.

[Para 4] Interferons (IFNs) attenuate RSV replication and also have therapeutic value against allergic diseases, including asthma 20-22. Further, we and others have developed in vivo intranasal gene delivery approaches using nanoparticles composed of chitosan, a natural, biocompatible, and biodegradable polymer21-24. Since bovine and human RSV NS1 appear to antagonize the Type-I interferon-mediated antiviral response25-28, it was reasoned that blocking NS gene expression might attenuate RSV replication and provide an effective antiviral and immune enhancement therapy. The short interference RNA (siRNA) approach has proven effective in silencing a number of genes of different viruses29. This approach was utilized herein to examine the potential and mechanism of siRNA against NS1 (siNS1) to inhibit RSV replication in cultured human epithelial cells, to modulate immunity to RSV in human dendritic cells, and to attenuate RSV infection in mice. The results demonstrate that siNS1-mediated

silencing of the NS1 gene significantly suppresses RSV replication and modulates host immunity to RSV compared with control groups, suggesting that the NS genes are suitable targets for RSV prophylaxis.

[Para 5] SUMMARY OF INVENTION

[Para 6] Respiratory syncytial virus (RSV) infection is one of the major causes of respiratory tract infection in all age groups, for which no vaccine or antiviral is available. RSV NS1 protein appears to antagonize antiviral host-IFN response, however, its mechanism is unknown. Herein, a plasmid-borne small interference RNA-targeting the NS1 gene (siNS1) was used to examine the role of NS1 in modulating RSV infection. siNS1-transfected A549 epithelial cells exhibit decreased NS1 protein expression and RSV titers and up-regulated expression of IFN-inducible genes, such as STAT1 and IRF-1/3. Also, siNS1-transfected human dendritic cells upon RSV-infection produce elevated type-I IFN and induce differentiation of naïve CD4+ T cells to Th1 cells. Mice treated intranasally with siNS1-nanoparticles prior to or after infection with RSV show significantly decreased virus titers in the lung and decreased inflammation and airway reactivity compared to control. Thus, siNS1-nanoparticles may provide effective prophylaxis against RSV infection of humans.

[Para 7] FIGURE LEGENDS:

[Para 8] Fig. 1. siNS1 inhibits the replication of rgRSV. a, Immunoblot analysis of NS1 protein expression. The proteins from whole infected cells were immunoblotted with a RSV polyclonal antibody and actin (control). b,c, Flow cytometry analysis of rgRSV positive A549 cells and Vero cells, respectively. The numbers of RSV-infected cells were determined by flow cytometry as EGFP density. d, e, Measurement of virus titer. Virus titers in the supernatant either from infected A549 cells and Vero cell were determined by plaque assay on A549 cells. Data are the averages of two independent experiments. **$p<0.01$ when compared with control group.

[Para 9] Fig. 2. siNS1-mediated attenuation of RSV infection involves up-regulated expression of IFN-β and IFN-inducible genes in infected A549 cells. a.The proteins from whole infected cells were immunoblotted with appropriate antibody to IFN-β and β-actin (control). c, Immunoblot analysis of the expression of IFN-inducible genes in RSV-infected cells. b, d, Protein bands were scanned using the Scion image system (NIH) to quantitate data from a and c, respectively.

[Para 10] Fig. 3. NS1 protein prevents nuclear import of IRF1 and STAT1. a, IRF1 and STAT1 proteins subcellular distribution were examined by indirect immunofluorescence using corresponding antibody. *$p<0.05$ and **$p<0.01$ relative to control. b, Quantification of phopho-STAT1 and IRF1 positive A549 cells transfected with siNS1 or control siRNA prior to inoculation. Results of one representative experiment of three are shown.

[Para 11] Fig. 4. Effect of siNS1 on human DCs. a, Expression levels of IFN-α and IFN-β protein in DCs infected with RSV were measured by ELISA assay. b, Flow

cytometric analysis of intracellular cytokine production in allogeneic naïve CD4+ T cells. Naïve CD4+ T cells isolated from cord blood were co-cultured with RSV-infected DCs, treated with or without siNS1, which had been stained with IFN-γ-FITC and IL-4-PE antibodies for flow cytometry analysis. Bar graph showing the quantitative of % gated cells. Results are shown from three separate experiments.

[Para 12] Fig. 5. Antiviral and anti-inflammatory activity of siNS1in vivo. a, Detection of NS1 gene expression. The total RNAs were extracted from homogenized lungs at 18 h post-infection with rgRSV and NS1 gene expression was determined using RT-PCR. b, Determination of viral lung titer. Mice (n=8) were sacrificed and viral titers in lung homogenates were measured by plaque assay on A549 cells. *$p<0.05$ relative to control. c, Airway responsiveness of rgRSV-infected mice treated with NG042-plasmid complex (2 days prior to rgRSV infection). Airway responsiveness to inhaled methacholine (MCh) was measured and the results are expressed as % Penh (enhanced pause) after inhalation of MCh relative to PBS. *$p<0.05$ compared to control. d, Histology of lung sections (2 days prior to rgRSV infection). Mice were sacrificed, lungs were removed, and the histologic sections were stained with H&E. e, Time course of prophylaxis. Mice (n=6) were administered Nano-siNS1 2, 4 or 7 d prior to RSV infection. They were sacrificed 5 d postinfection and viral titers in lung homogenates were measured by plaque assay on A549 cells. *$p<0.05$ relative to control.

[Para 13] Fig.6. Nano-siNS1 decreases established rgRSV infection. a, Determination of viral lung titer. Mice (n=8) were sacrificed and viral titers in lung homogenates were measured by plaque assay on A549 cells. *$p<0.05$ relative to control. b, Histology of lung sections (plasmid complex used at day 2 postinfection of rgRSV). Mice were sacrificed, lungs were removed, and the histologic sections were stained with hematoxylin and eosin (H&E). Results of one representative experiment of two are shown.

[Para 14] Fig. 7. Nano-siNS1 decreases reinfection and alters cytokine profiles in rgRSV-infected mice. a, Measurement of viral lung titer from reinfection mice (1x 107 PFU/mouse) at day 21 after primary infection. *$p<0.05$ compared to control. b, c, Flow cytometric analysis of intracellular cytokine production in spleen T cells. CD4+ and CD8+ T cells isolated from spleen cells were stained either with IFN-γ-PE or IL-4-PE and CD4- or CD8-FITC antibodies and analysed by flow cytometry analysis. Bar graphs show the quantitation of % gated cells. Results shown are average of three separate experiments.

[Para 15] DETAILED DESCRIPTION OF PREFERRED EMBODIMENT OF INVENTION

[Para 16] siNS1 inhibition of rgRSV replication involves IFN-pathway.

[Para 17] A plasmid vector, pSMWZ-1, was constructed with a mouse U6 promoter linked to a siRNA cassette to establish a siRNA system30. RSV NS1 or HPV18E7 siRNA oligos were designed and cloned into pSMWZ-1 to produce the siNS1 and siE7

plasmids, respectively. Analysis of EGFP expression in A549 cells cotransfected with pEGFP and siNS1 or siE7 demonstrates that siNS1 or siE7 did not silence the EGFP gene (data not shown). To examine whether siNS1 could attenuate virus replication, A549 cells were transfected with the siNS1 or siE7 constructs and infected with rgRSV, a recombinant RSV expressing enhanced green fluorescence protein (EGFP) 31. Cell lysates were analyzed for NS1 protein expression one day post-infection. A549 cells pre-transfected with siNS1, but not siE7, show a significant reduction in the expression of NS1 proteins (Fig. 1a) but not of other viral proteins (data not shown).

[Para 18] The effect of siNS1 on rgRSV replication was examined in A549 and Vero cells to determine whether the siNS1-mediated decrease of viral titers is dependent upon the Type-I IFN pathway. Vero cells are defective in the Type-I IFN pathway 32. The percentage of rgRSV-infected cells expressing EGFP decreases in a dose-dependent and sequence-specific manner in A549 (Fig. 1 b), not Vero (Fig. 1 c) cells. Furthermore, plaque assays for RSV titers in culture supernatants indicate that transfection with siNS1 significantly decreases rgRSV titer compared to control ($p<0.01$) in A549 (Fig. 1d), not Vero cells (Fig. 1e). These results indicate that NS1 affects type I IFN pathway.

[Para 19] NS1 regulates production of IFN and IFN-stimulated genes in epithelial cells.

[Para 20] NS1 proteins appear to exert antagonistic effects on Type-I IFNs27, 28; however, the mechanism remains unclear. To determine whether NS1 decreases the amount of type-I IFN, cells transfected with siNS1 and the control siRNA were examined by immunoblotting for IFN-β production. Results show that siNS1 significantly increases IFN-β production compared to controls (Fig. 2 a, b). To further verify the role of NS1 in regulating the IFN pathway, RNAs from control and siNS1-transduced cells were isolated and subjected to microarray analyses. The results show that siNS1 treatment increases the expression (≥6 fold-changes) of 25 IFN-inducible genes compared to rgRSV infection alone (Table 1). To confirm the microarray data, the expression of a number of altered genes was investigated by western blotting. The results show that the pSTAT1 (Ser 727), STAT1, IRF1, IRF3, IRF7, ISGF-3γ and MxA proteins were up-regulated after siNS1 inhibition. (Fig. 2 c, d). A densitometric analysis shows that the proteins that exhibit the maximal increase in expression following siNS1 transfection include STAT1, IRF1, MxA, ISGF-3γ and IFN-β (Fig. 2b, d).

[Para 21] NS1 blocks nuclear translocation of IRF1 and phosphor-STAT1 proteins.

[Para 22] The subcellular distribution of the IRF1 and pSTAT1 proteins in the infected A549 cells following siNS1 transduction and RSV infection was examined further using indirect immunofluorescence to determine whether NS1 affects STAT1 and IRF1 translocation. Thus, transfected-cells were infected with the wild-type RSV (MOI=0.1), fixed 3 h later, permeabilized, and stained with appropriate antibody. The IRF1 and pSTAT1 proteins both exhibit more characteristic nuclear localization patterns after

siNS1 treatment (Fig. 3a, 3b). The subcellular distribution of the IRF1 and pSTAT1 proteins differs among experimental groups, suggesting that the NS1 protein blocks trafficking of these proteins into the nucleus.

[Para 23] Silencing NS1 polarizes human dendritic cells toward a Th1-promoting phenotype

[Para 24] While epithelial cells are the major target cells for virus replication, dendritic cells (DCs) play a significant role in mounting the immune response to RSV infection. Monocytes isolated from human peripheral blood were cultured with requisite cytokines to test whether siNS1 expression affects DC activity. Thus, the IFN-α and -β concentrations were measured in the supernatants from cultured, infected, monocyte-derived DCs transfected with siNS1 or control. The data shows that siNS1 treatment induces a significantly higher production of both Type-I IFNs in infected DCs than in controls (Figure. 4a). Furthermore, to assess the effect of siNS1-treated DCs on T-cell function, allogeneic naïve CD4+ T cells were co-cultured with RSV-infected DCs treated with or without siNS1. Intracellular IFN-γ and IL-4 secretion in naïve CD4+ T cells was assessed by intracellular cytokine staining. The results demonstrate that siNS1-treated, RSV-infected DCs increase IFN-γ and decrease IL-4 production in naïve CD4+ T cells compared with controls (Fig. 4b).

[Para 25] Prophylaxis with nanoparticle-complexed siNS1(Nano-siNS1) significantly attenuates RSV infection and pulmonary pathology in mice.

[Para 26] While the vector-driven siRNA approach is effective in cell cultures, its in vivo application remains to be elucidated. To determine whether siNS1 exerts an antiviral response in vivo in BALB/c mice, the siNS1 plasmid was complexed with Nanogene 042 (NG042) nanoparticles and administered as a nasal drop 2 d before viral inoculation. NS1 expression in the lungs (n = 6 for each group) of mice was assayed by RT-PCR 18h post-infection. As revealed by RT-PCR data, siNS1 significantly knocked down expression of the RSV-NS1 gene but not of the RSV-F gene (Fig. 5a). The viral titer in supernatants of homogenized lungs (n = 8) was measured by a plaque assay on A549 cells. The results indicate that siNS1 significantly decreases viral titers in the lungs of infected mice ($p<0.05$) (Fig. 5b). These mice (n=8) were challenged with methacholine at day 4 following rgRSV infection. RSV-infected mice showed a greater than 400% increase in enhanced pause (Penh) values compared to baseline and a 300% increase compared to the siNS1 group (Fig. 5c). Mice treated with siNS1 show significantly lower AHR than that of untreated mice ($p<0.05$) and exhibit a significant reduction in the pulmonary inflammation as evidenced by decreases in the goblet cell hyperplasia of the bronchi and in the number of infiltrating inflammatory cells in the interstitial regions compared to controls (Fig. 5d). To investigate the persistence of siNS1 prophylaxis, mice were treated with the siNS1-Nanogene complex for 2, 4 and 7 days prior to the viral inoculation. Analysis of viral titers 5 d post-infection shows that NG042-siNS1 can induce a significant reduction of

viral titers when given 4 d prior to RSV inoculation, although treatment on day -7 lowered viral titer by 10-fold compared to control (Fig. 5e).

[Para 27] Potential of Nano-siNS1 for treatment of RSV infection.

[Para 28] Early treatment with antivirals is important in the clinical realm to attenuate severity of primary RSV infection. To test the therapeutic potential of Nano-siNS1 mice were administered with Nano-siNS1 at day 0 along with RSV infection. The results show that treated mice exhibit a significantly lower (3 log) viral titer compared to controls ($p<0.05$) (Fig. 6a). To determine whether Nano-siNS1 treatment can attenuate ongoing RSV infection, mice were infected with RSV and then treated the Nano-siNS1 after days 1, 2, or 3 of viral inoculation. The viral titers measured in these mice show that treatment with Nano-siNS1 complex at 2 d post-infection of RSV significantly decreases viral titer ($p<0.05$) and reduced mice lung inflammation (Fig. 6a, 6b). Treatment with Nano-siNS1 at 3 d post-inoculation also decreases virus titer, albeit marginally.

[Para 29] Nano-siNS1 augments anti-RSV immunity.

[Para 30] Prophylaxis or treatment with siNS1decreases NS1 expression without affecting other RSV genes at the protein level in cells (data not shown) and RNA level in mice (Figure 5a). To test whether NS1 plays a role in the anti-RSV immunity, protection from reinfection and functional T cell response was examined. Results show that a single dose of siNS1 prevents mice from reinfection with a higher dose of RSV 16 d after the primary RSV challenge (Fig. 7a). Spleen T cells isolated from the reinfected mice 5 days later were assessed for IFN-γ and IL-4 secretion in CD4+ and CD8+ T cells using intracellular cytokine staining. The results demonstrate that siNS1-treated mice increase IFN-γ production in both CD4+ and CD8+ T cells and also increase IL-4 production in CD4+ T cells compared with controls (Fig. 7 b, c). Thus, intranasal administration of siNS1 prior to viral infection not only knocks down RSV infection at an early stage but also enhances both cellular and humoral immune responses for attenuation of RSV reinfection.

[Para 31] DISCUSSION

[Para 32] Although the human RSV NS1 protein has a Type-I IFN-antagonistic effects, the mechanism remains unknown. This report describes, for the first time, the significant role of NS1 in RSV replication and immunity to RSV infection. These studies demonstrate that the NS1 protein downregulates the IFN-signaling system by deactivation of STAT1, IRF1, and IFN-regulated gene expression, which are critical to suppressing IFN action. Furthermore, the results reveal the potential for nanoparticles encapsulating siNS1 for the prophylaxis and treatment of RSV infections.

[Para 33] Vector-driven de novo expression of siRNA to attenuate RSV infection has not been reported heretofore, although antisense oligonucleotide-mediated attenuation of RSV infection in African Green Monkeys was reported33. However, the potential of this approach remains uncertain as the effects of these oligos were measured at the

very early stage of infection, i.e., 30 min post-RSV challenge. Mechanistically, both antisense and siRNA work at the post-transcriptional level to reduce the expression of the target gene. The antisense oligonucleotides accumulate in the nucleus and may alter splicing of precursor mRNA34. In contrast, siRNAs exert function in the cytoplasm35,36, which is the site of RSV replication. Also, intracellular expression from RNA polymerase III promoters enables the production of stably expressing siRNA in cell with sustained knockdown of the target and hence, lower concentrations are needed to achieve levels of knockdown that are comparable to antisense reagents.

[Para 34] A major finding of this report is the demonstration that DNA-vector driven siNS1 expression is capable of significantly attenuating the RSV infection of human epithelial cells, which are the primary targets of RSV replication. A549 epithelial cells were used, as they are similar to cultured primary airway cells in terms of their susceptibility to RSV37. The transfection efficiency of the construct using plasmid pEGFP was 43.21% and 49.62% in A549 and Vero cells, respectively. Despite this, the siNS1 plasmid inhibited rgRSV production by 90-97%, which is consistent with a 2 to 3 log decrease in RSV titers. Although the mechanism of the siNS1-mediated decrease in viral titers was not investigated, it may be attributed to the fact that NS1, located at the 3′ end of the viral genome, acts as a common early stage promoter for the initiation of both replication and transcription38. These results are consistent with reports, which suggest that deletion of NS1 strongly attenuates RSV infection in vivo 12, 14, 15 and implicate the potential application of siNS1 for prophylaxis against RSV infection.

[Para 35] The mechanism of siNS1-induced attenuation of viral replication was investigated. To establish that the antiviral and anti-inflammatory effects of siNS1 are due to IFN, Vero cells that lack the Type-I IFN genes were either cotransfected with pEGFP and siEGFP or transfected with siNS1. siEGFP significantly knocked down EGFP gene expression (91.68%); however, siNS1 did not significantly affect either the percentage of infected Vero cells or the viral titer, suggesting that siNS1 effects involve the production of Type-I IFN. These results are in agreement with the increases in IFN production observed with NS1/NS2-deleted human RSV infection25-28.

[Para 36] Plasmid-driven siRNA induces IFN and IFN-stimulated genes, including PKR and OAS39,40. The IFN production by siRNA may be attributed to CpG motifs (ampr gene) present in the vector plasmid that activate innate immunity via binding to TLR941. It is also likely that U6 vector used in these studies contribute to the IFN production, as the U6 vector induces a higher frequency of interferon-stimulated genes compared to lentiviral H1 vectors42. In this study, siNS1 induced significantly higher amounts of IFN compared to siE7 (the same vector as siNS1), indicating that NS1 is involved in antagonizing Type-I IFN. In addition, the transfection of Vero cells with siE7 did not attenuate viral infection, suggesting that the plasmid itself might induce transfected A549 cells to up-regulate certain IFN-inducible genes. This could account

for the finding that siE7 or siPB2 somewhat reduced rgRSV production in vitro or in vivo and that siE7 and empty vector induced IFN-β in A549 cells.

[Para 37] IFNs drive a cascade of intracellular signaling, resulting in the expression of a large number of interferon-stimulated genes (ISGs) that exert the pleiotropic effects of IFN, including interference with viral replication and modulation of the host immune response43. The level of expression of IFN-inducible genes in infected A549 cells treated with siNS1 was significantly altered, as revealed by the microarray data. IRF3 and MxA expression were upregulated after NS1 inhibition, in agreement with a previous report26, although STAT2 levels were not changed. Other genes, including STAT1, pSTAT1 (Ser 727), IRF1, and ISGF-3γ, were also upregulated compared to control. These differences may be attributed to the fact that human RSV and different cell lines were utilized. These data indicates that IRF1, IRF3 and ISGF-3γ function as transcription activators44. IRF1 binds to the positive regulatory domain 1 (PRD1) of the IFN-β promoter45 and to the IFN-stimulated response element (ISRE) of IFN-stimulated genes46. ISGF-3γ encodes a protein-interaction function that allows recruitment of STAT1 and STAT2, their translocation from the cytoplasm to the nucleus, and initiation of transcription of IFN- stimulated genes (ISGs) 43. These data show that more IRF1 and pSTAT1 proteins translocate into the nucleus of infected A549 cells through knockdown of the NS1 protein suggesting that NS1 targets activation of STAT1 and IRF1.

[Para 38] Whereas epithelial cells are the major target cells in which the virus replicates, monocytes and dendritic cells play a role in generating anti-RSV immunity. Monocytes play a role in the pathophysiology of RSV bronchiolitis47, and they represent a pool of circulating precursors capable of differentiating into DCs that are able to present pathogen-derived peptides to naïve T cells. NS1 appears to decrease type-1IFN production in DCs, presumably affecting their state of activation and antigen presentation. These studies also demonstrate that RSV infection decreases the capacity of DCs to induce IFN-γ in naïve T cells 48, which might cause the delayed RSV-specific immune response and permit multiple RSV reinfections. The results of this study demonstrate that infected DCs treated with siNS1 produce much more Type-I IFN and also drive naïve CD4+ T cells toward Th1-type lymphocytes that generate more IFN-γ and less IL-4.

[Para 39] The effects of siRNA have been amply demonstrated in cultured cells, however, only a few studies have addressed the potential of siRNA-based therapeutics in vivo using model animal systems. To test therapeutic potential of siNS1, a new generation of Nano-size chitosan, such as NG042, was used for de novo expression of siNS1 in the nasal tissue. NG042 shows higher transduction efficiency and induces less inflammation compared to classical chitosan (unpublished data). The results of studies on the prophylactic potential of Nano-siNS1 indicate that siNS1 can induce significant protection from rgRSV infection, infection-induced inflammation, and airway reactivity, and the protective effect lasts for at least 4 days. Furthermore, even a single dose of

Nano-siNS1 intranasal administration can prevent mice from reinfection with a higher dose of RSV 16 d after primary infection. The precise mechanism of enhanced protection is unknown, but it is likely that knockdown of the NS1 gene augments anti-RSV host immunity via enhanced IFN production and thereby prevents mice from RSV reinfection. In addition, Nano-siNS1 also attenuates the established RSV infection. Thus, the antiviral treatment decreased viral titer in the lung, improved pulmonary function, and attenuated pulmonary inflammation in rgRSV-infected mice. It is noteworthy that the results of studies of RSV infection in Fisher rats using Nano-siNS1 are similar to that seen in mice (Mohapatra and Piedemonte, unpublished data) further suggesting their potential.

[Para 40] In conclusion, these data suggests that NS1 appears to possess both antiviral and immunomodulatory roles which determines the magnitude viral replication and severity of infection. The antiviral effect of the siRNA-inhibition of RSV NS1 gene expression is attributable both to blocking viral replication and to preventing the down-regulation of IFN-inducible genes. Also, Nano-siNS1 may prove to be a potent, new prophylactic and/or therapeutic agent against RSV infection in humans.

[Para 41] METHODS

[Para 42] Virus and cell lines. A549, Vero cell line and RSV strain A2 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in complete DMEM. Recombinant rgRSV which encodes green-fluorescent protein was kindly supplied by Dr. Mark E. Peeples31.

[Para 43] Plasmid constructs. The nucleotide sequence for each siRNA is as follows: siNS1: 5′-GGCAGCAATTCATTGAGTATGCTTCTCGAAATAAGCATACTCAATGAATTGCTGCCTTTTTG-3′; siE7: 5′-GAAAACGATGAAATAGATGTTCAAGAGACATCTATTTCATCGTTTTCTTTTTT-3′; siPB2: 5′-GGCTATATTCAATATGGAAAGAACTCGAGTTTTGTTCTTTCCATATTGAATATAGCCTTTTTG-3′; Each pair of oligos was annealed and inserted into pSMWZ-1 plasmid at appropriate sites,

[Para 44] respectively to generate the corresponding siRNA for RSV NS1, HPV18 E7 and type A Influenza virus PB2.

[Para 45] DNA transfection and virus infection. Cells were transfected with siNS1 using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The cells were then infected with rgRSV or RSV at appropriate multiplicity of infection. pEGFP plasmid (Stratagene, La Jolla, CA) was used for transfection ratio measurement.

[Para 46] Isolation of DCs from human peripheral blood and measurement of IFNs in supernatants of infected DCs. Monocytes were purified from PBMCs using monocyte isolation Kit II (Miltenyi Biotec., Auburn, CA). Lastly all the monocytes were seeded into six-well culture plates supplemented with 10% FBS (Cellgro, Herndon, VA), 200ng/ml IL-4 and 50ng/ml GM-CSF (BD-Pharmingen, San Diego, CA)

and cultured for 6-7 days for plasmid transfection and infection with RSV. Expression level of IFNs in the supernatants was assayed by IFN-α Multi-Species ELISA Kit and IFN-β ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ).

[Para 47] Analysis of intracellular cytokine production in T cells. Human naïve CD4+ T cells were purified using CD4+ T cell isolation kit (Miltenyi Biotec. Auburn, CA) from umbilical cord blood. And Naïve CD4+ T cells (1X106 cells/well) were co-cultured with irradiated monocyte-derived DCs (30 Gy) (1X105 cells/well) in 24-well plates for 6 days with additional culture for 8 days in the presence of recombinant hIL2 (10ng/ml); Mice spleen T cells were purified using mouse T-cell enrichment column kit (R & D Systems, Minneapolis, MN), and the T cells were cultured in 6-well plates for 4 days. Finally, all the cells were stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) (Sigma, Saint Louis, Missouri) for 6 h in the presence of GolgiStopTM (PharMingen, San Diego, CA) and then fixed and stained either using FITC -anti-IFN γ mAb, PE -anti-IL4 mAb and FITC-anti-CD4 or –CD8 mAb (BD Biosciences, San Diego, CA) for flow cytometry analysis.

[Para 48] Flow cytometry. The cells were harvested 1 day post-infection, washed with PBS twice, resuspended with 1% paraformaldehyde and kept on ice for 30min. The numbers of rgRSV infected cells were quantified by flow cytometry.

[Para 49] Immunofluorescence. A549 cells growing on 8-well chamber (Nalge Nunc Int., Rochester, NY) were processed for immunofluorescence at room temperature. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 3% Donkey serum in PBS containing 1% Glycerin for 60 min. Cells were next incubated with IRF1 antibody (Santa Cruze Biotec., Santa Cruze, CA) or pSTAT1 (Ser 727, Upstate, Charlottesville, VA), respectively, and then with Zenon TM Alexa Fluor® 488 (Molecular probes, Eugene, OR). The slides were visualized by immunofluorescence microscopy.

[Para 50] Plaque assay. 10-fold serial dilutions of the supernatants were added to a monolayer of A549 cells and the medium in each well of six-well culture plates was replaced by an agarose-containing overlay (2X DMEM, 10% FBS, 1% Low Melting Point Agarose (Gibco BRL, Rockville, MD). The plates were incubated at 37 °C for 5 days. Afterwards, 1% Neutral red (Sigma, Saint Louis, Missouri) was added and the plaques were counted 48h later.

[Para 51] Microarray assays. Total RNAs were extracted by RNase® (Qiagen RNeasy Kit). 10μg of total RNAs were used to prepare cDNA. Gene expression was analyzed with GeneChip® Human Genome U95Av2 probe array (Affymetrix, Santa Clara, CA) according to the manufacture's protocol (Expression Analysis Technical Manual). Data analysis was performed with Microarray Suite 5.0 (MAS 5.0).

[Para 52] Protein expression analysis by western blotting. Transfected A549 cells were infected with rgRSV (MOI=1). The whole cell lysates were electrophoresed on 12% polyacrylamide gels and the proteins were transferred to PVDF membranes

(BIO-RAD, Hercules, CA). The blot was incubated separately with RSV polyclonal antibody (AB1128, Chemicon Int. Temecula, CA), STAT1, pSTAT1 (Tyr 701), STAT2, IRF1, IRF3, IRF7, ISGF-3γ and IFN-β (Santa Cruze Biotech., Santa Cruze, CA), pSTAT1 (Ser 727, Upstate, Charlottesville, VA) or MxA antibody (Dr. Otto Haller, Germany). Immunoblot signals were developed by Super Signal Ultra chemiluminescent reagent (Pierce, Rockford, IL.).

[Para 53] Studies in mice. Six-week old female BALBl/c mice were purchased from Charles River Laboratory (Frederick, MD) and were maintained in a pathogen-free animal facility. BALB/c mice (n=8) were administered with plasmid with Nanogene-042 (a chitosan formulation, courtesy of TransGenex Nanobiotech, Inc, Tampa) intranasally (10μg/mouse of plasmid) prior to or after rgRSV inoculation (5X106 PFU/mouse). The pulmonary function was evaluated at day 4 post-infection as described previously (ref. 21). Finally, all mice were sacrificed the next day. The RSV titer was determined by plaque assay from the lung homogenate (n=8), and histological sections from lungs (n=8) were stained with hematoxylin and eosin.

[Para 54] Statistical analysis. Pairs of groups were compared by Student t test. Differences between groups were considered significant at $p < 0.05$. Data for all measurements are expressed as means ± SD.

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What is claimed is:

[Claim 1] A method of inhibiting respiratory syncytial virus infection comprising the steps of administering intranasal siRNA-nanoparticles of the NS1 gene.

ABSTRACT

Treatment intranasally with siNS1-nanoparticles prior to or after infection with RSV show significantly decreased virus titers in the lung and decreased inflammation and airway reactivity compared to control.

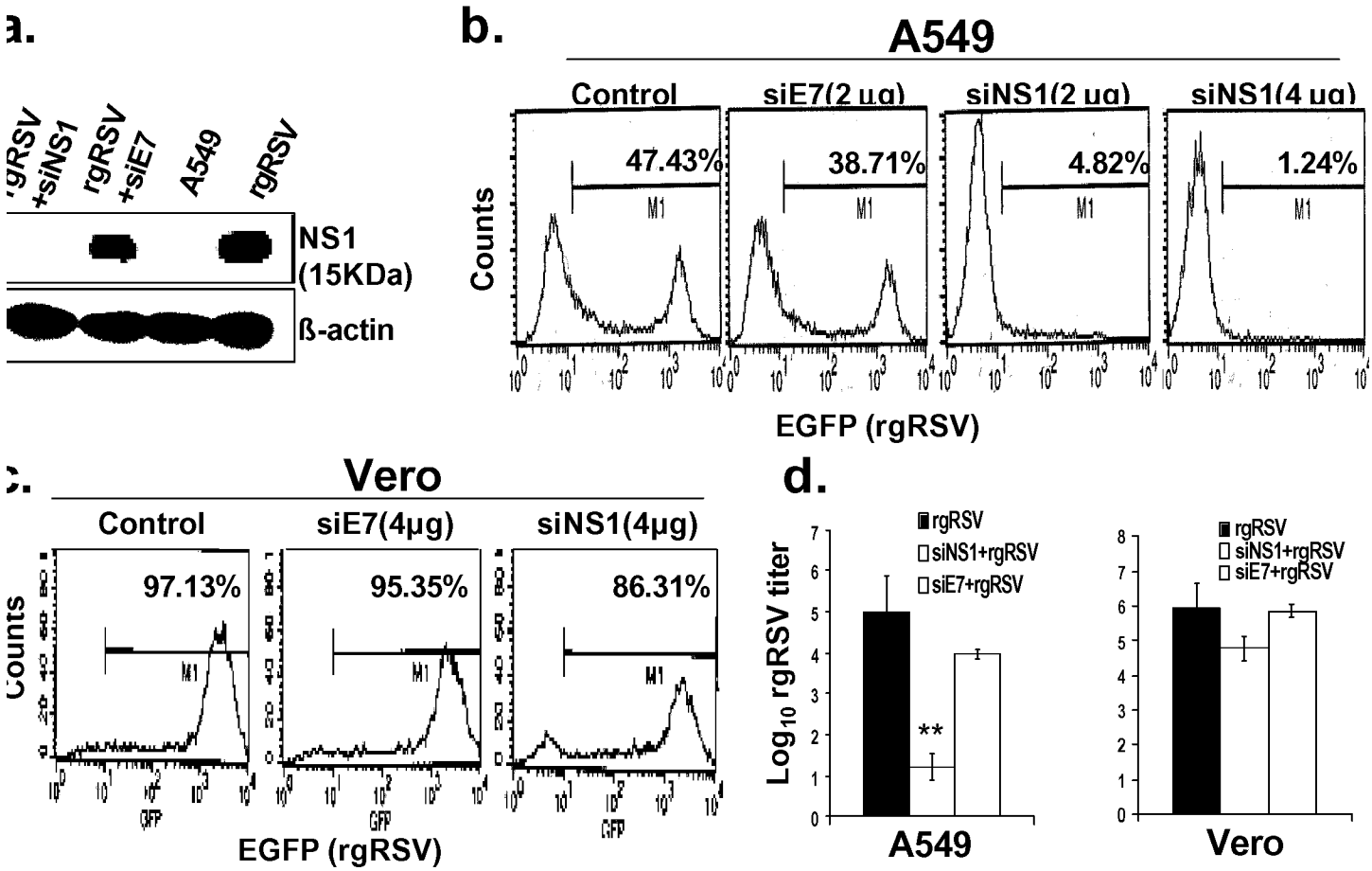

a.
rgRSV +siNS1
rgRSV +siE7
A549
rgRSV
NS1 (15KDa)
ß-actin
b.
A549
Control
siE7(2 ug)
siNS1(2 ug)
siNS1(4 ug)
47.43%
38.71%
4.82%
1.24%
M1
Counts
EGFP (rgRSV)
c.
Vero
Control
siE7(4μg)
siNS1(4μg)
97.13%
95.35%
86.31%
M1
Counts
GFP
EGFP (rgRSV)
d.
rgRSV
siNS1+rgRSV
siE7+rgRSV
Log10 rgRSV titer
**
A549
Vero

Fig.1

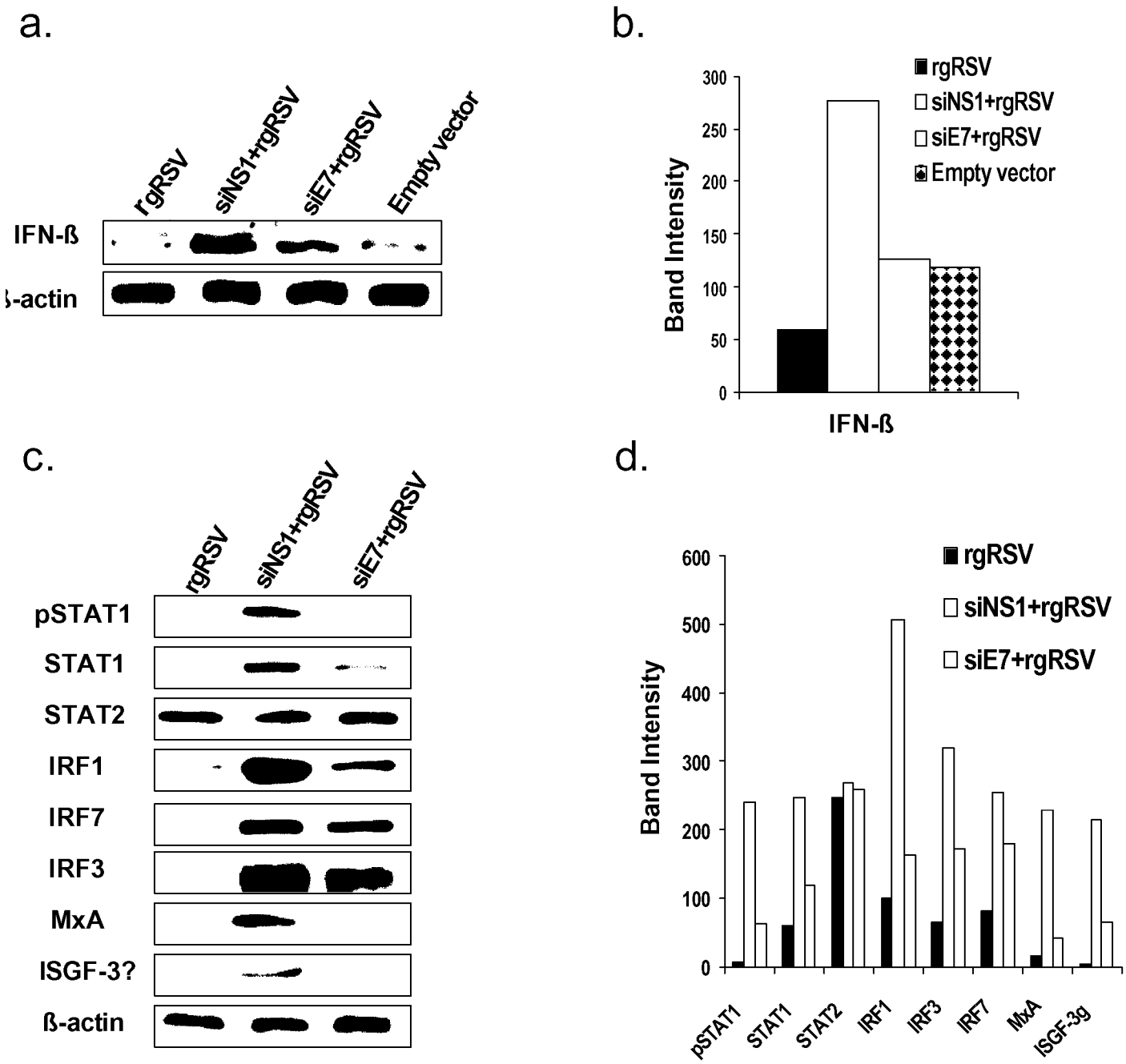
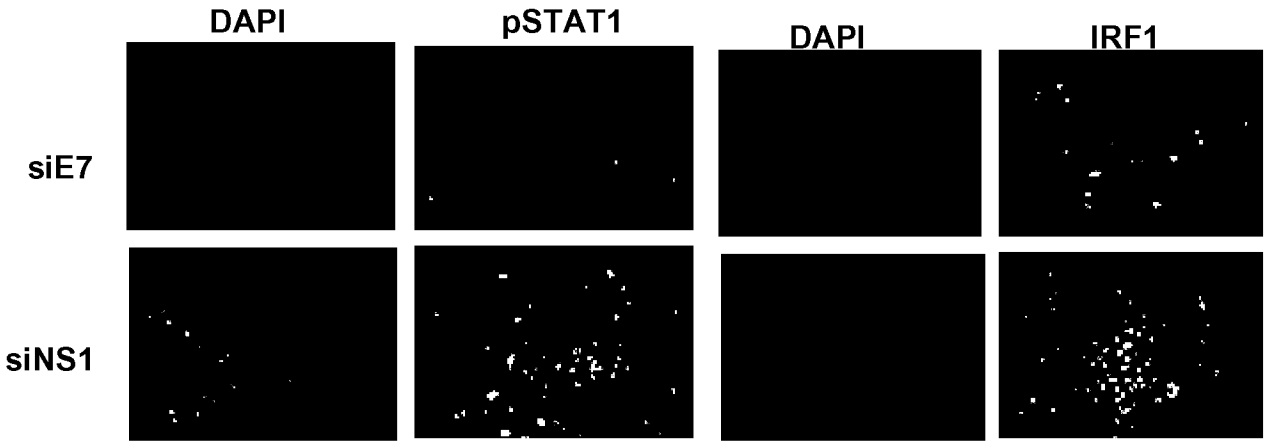

a.
rgRSV
siNS1+rgRSV
siE7+rgRSV
Empty vector
IFN-ß
ß-actin
b.
rgRSV
siNS1+rgRSV
siE7+rgRSV
Empty vector
Band Intensity
300
250
200
150
100
50
0
IFN-ß
c.
rgRSV
siNS1+rgRSV
siE7+rgRSV
pSTAT1
STAT1
STAT2
IRF1
IRF7
IRF3
MxA
ISGF-3?
ß-actin
d.
rgRSV
siNS1+rgRSV
siE7+rgRSV
Band Intensity
600
500
400
300
200
100
0
pSTAT1
STAT1
STAT2
IRF1
IRF3
IRF7
MxA
ISGF-3g

Fig. 2

a.


DAPI
pSTAT1
DAPI
IRF1
siE7
siNS1

b.

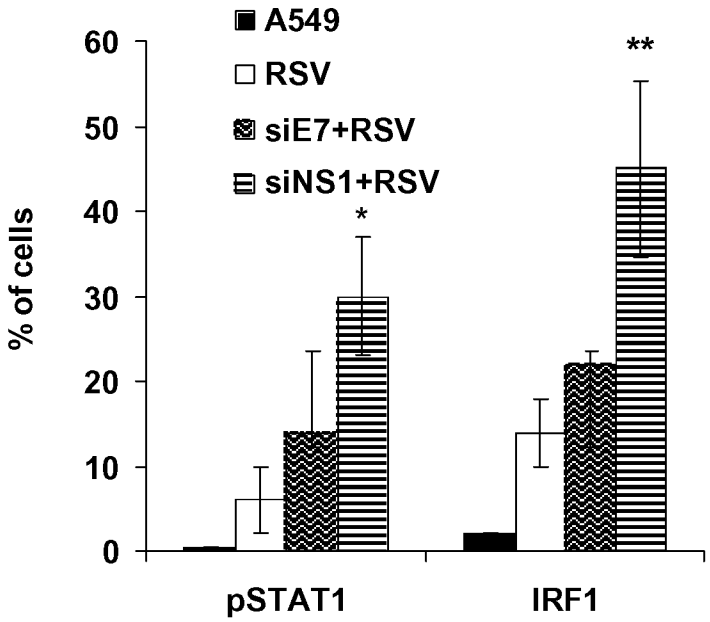

A549
RSV
siE7+RSV
siNS1+RSV
% of cells
60
50
40
30
20
10
0
*
**
pSTAT1
IRF1

Fig. 3

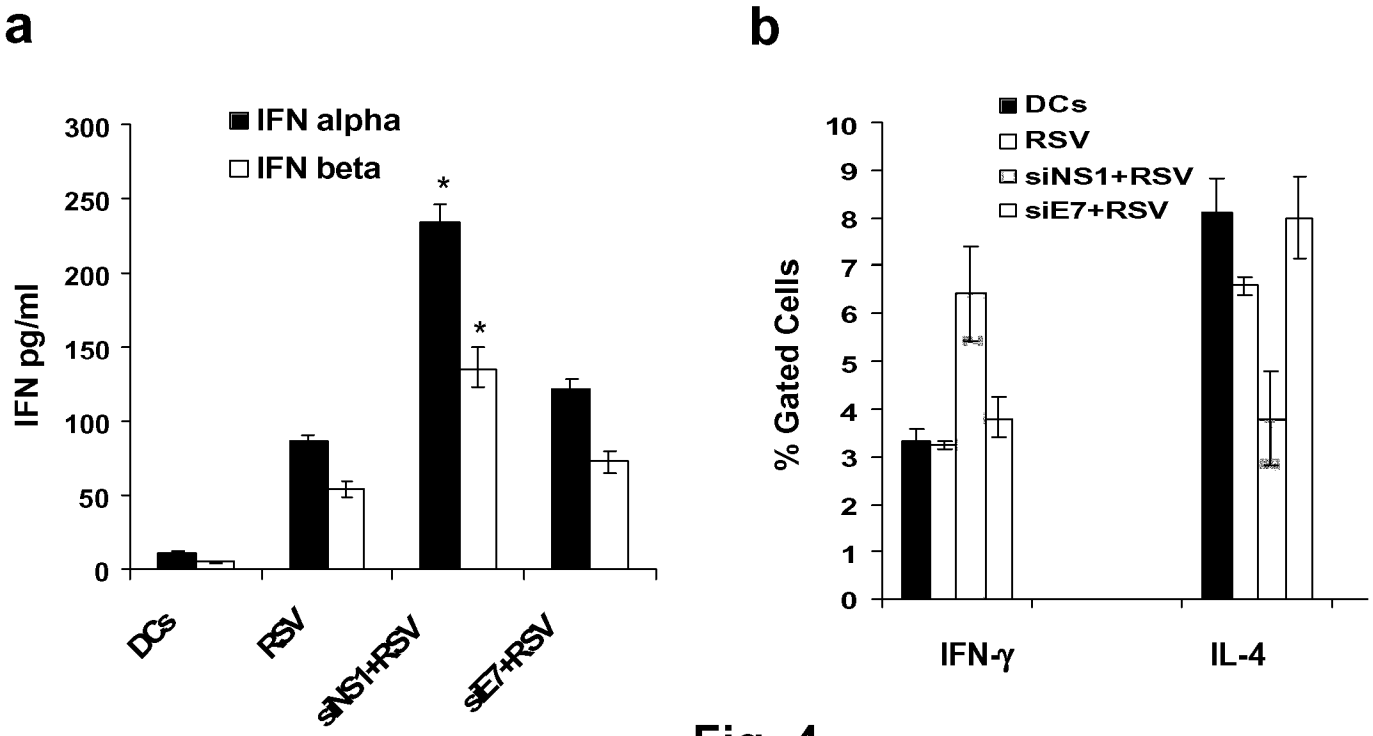

a
IFN alpha
IFN beta
IFN pg/ml
300
250
200
150
100
50
0
*
*
DCs
RSV
siNS1+RSV
siE7+RSV
b
DCs
RSV
siNS1+RSV
siE7+RSV
% Gated Cells
10
9
8
7
6
5
4
3
2
1
0
IFN-γ
IL-4

Fig. 4

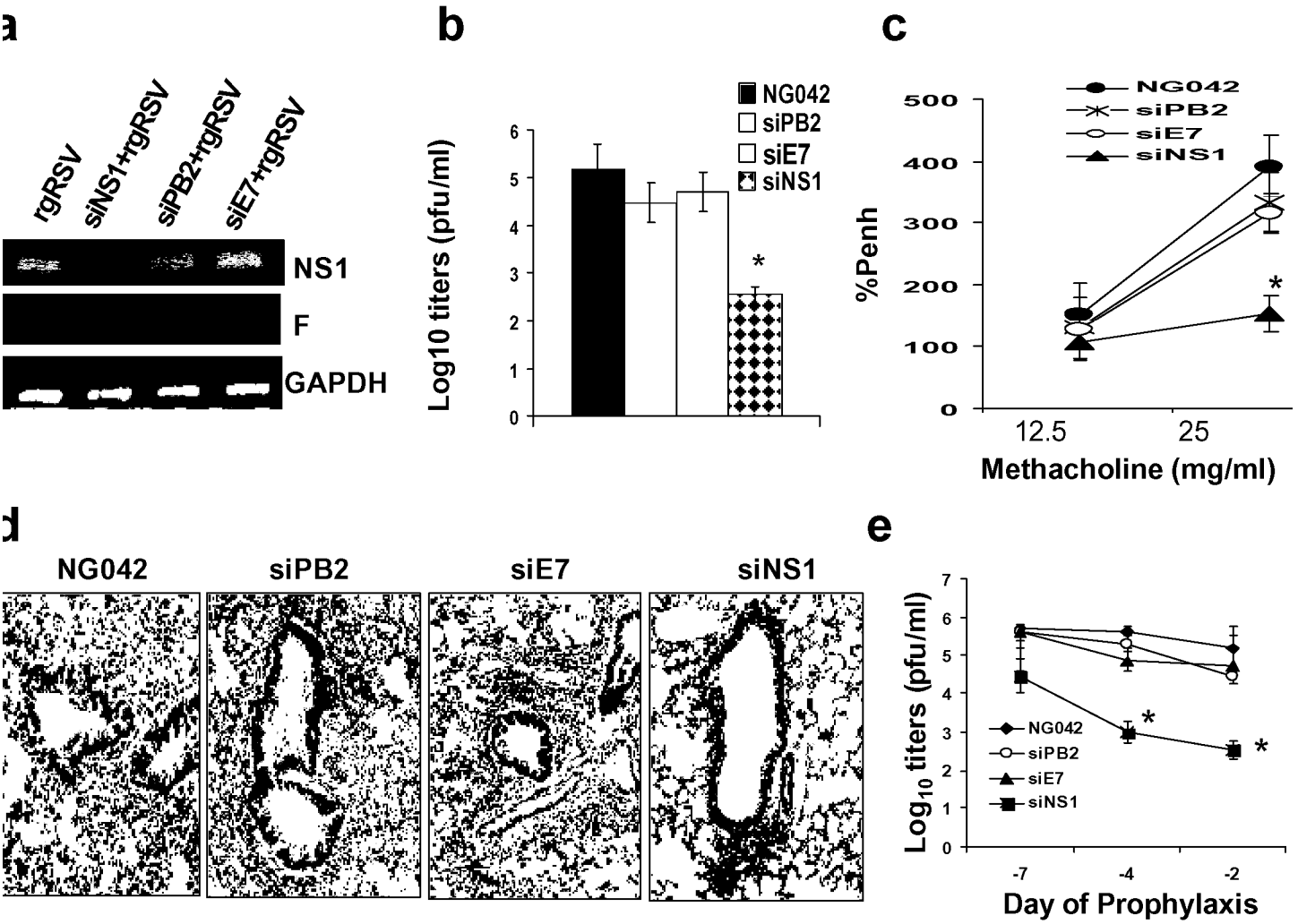

a
rgRSV
siNS1+rgRSV
siPB2+rgRSV
siE7+rgRSV
NS1
F
GAPDH
b
NG042
siPB2
siE7
siNS1
Log10 titers (pfu/ml)
*
c
NG042
siPB2
siE7
siNS1
%Penh
Methacholine (mg/ml)
12.5
25
d
NG042
siPB2
siE7
siNS1
e
Log10 titers (pfu/ml)
NG042
siPB2
siE7
siNS1
Day of Prophylaxis
-7
-4
-2

Fig. 5

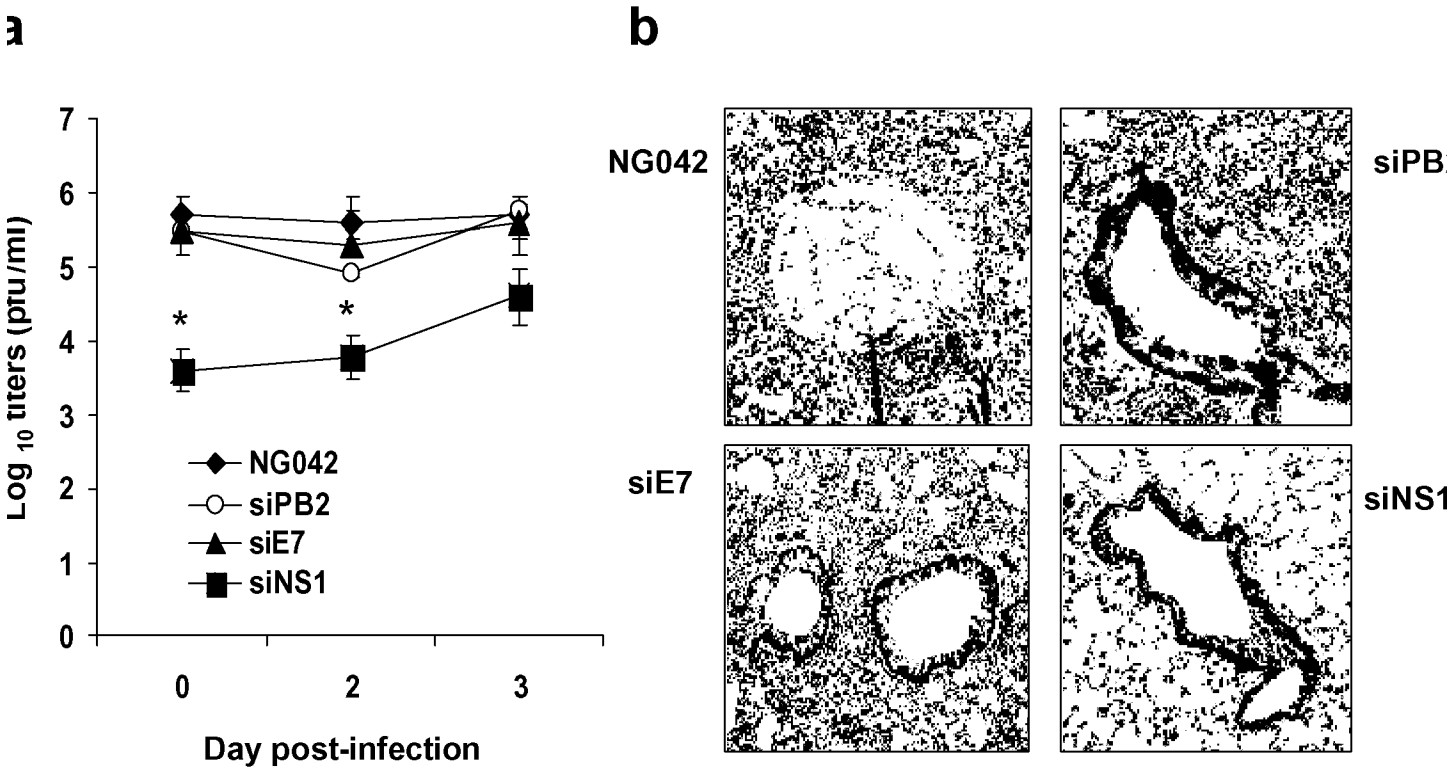

a
b
Log 10 titers (ptu/ml)
7
6
5
4
3
2
1
0
0
2
3
Day post-infection
*
*
NG042
siPB2
siE7
siNS1
NG042
siPB
siE7
siNS1

Fig. 6

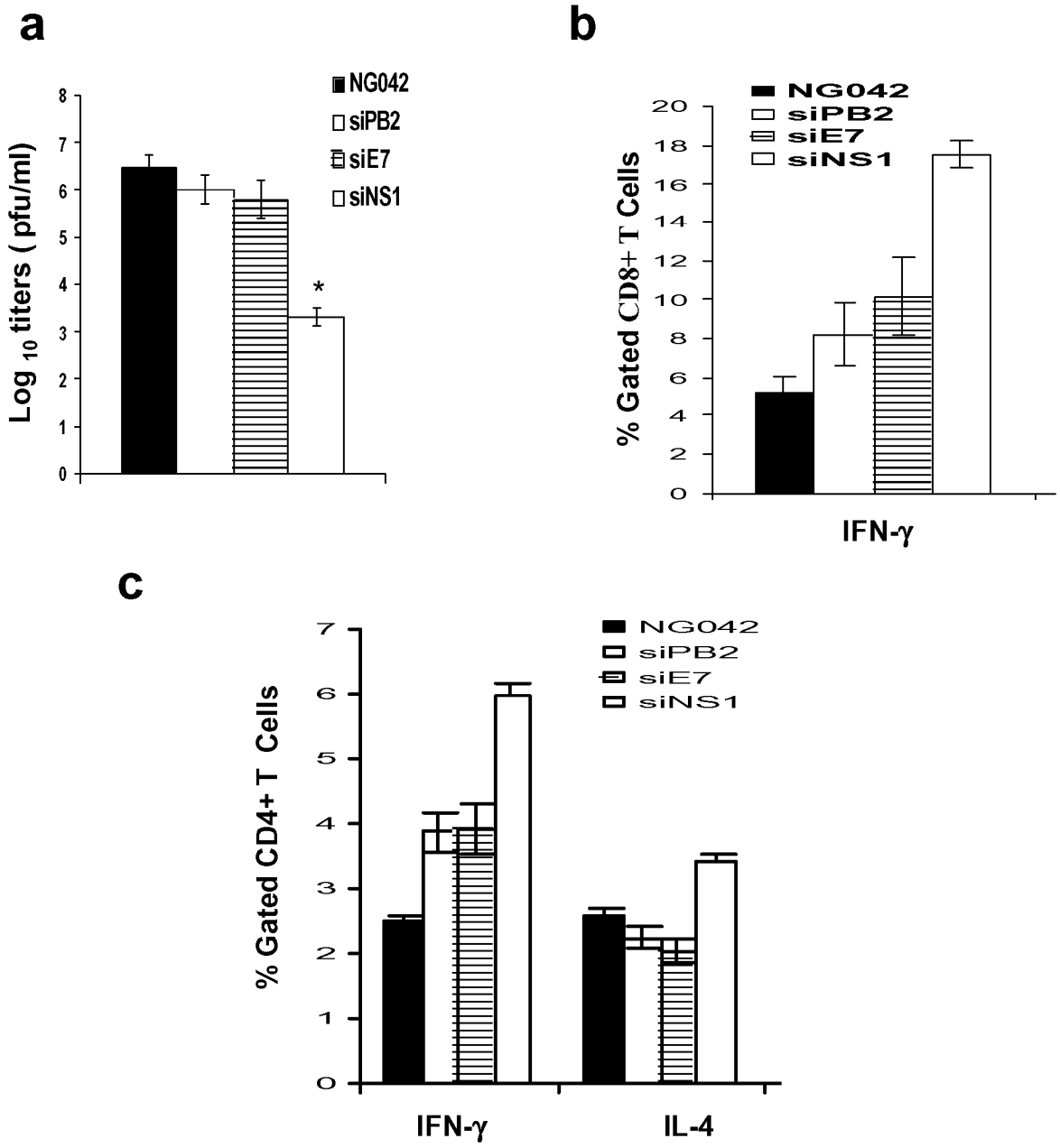

a
NG042
siPB2
siE7
siNS1
Log 10 titers (pfu/ml)
*
b
NG042
siPB2
siE7
siNS1
% Gated CD8+ T Cells
IFN-γ
c
NG042
siPB2
siE7
siNS1
% Gated CD4+ T Cells
IFN-γ
IL-4

Fig. 7